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Antigen expression in different parenchymal cell types of rat kidney and heart

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Antigen expression in different parenchymal cell types of rat kidney and heart. Rat glomerular epithelial, and mesangial and tubular cells were isolated by steel meshes of different pore sizes and enzymatic treatment. Rat heart endothelial cells were isolated by enzymatic disaggregation. Endothelial, glomerular mesangial, glomerular epithelial and tubular cells were cultured in Minimum essential medium until they reached confluency (5 to 9 days). These different rat parenchymal cell types were characterized by morphology and antibody stainings. Endothelial cells were characterized by factor VIII positivity and mesangial cells were desmin positive. Both glomerular epithelial and tubular cells expressed the brush border and Tamm-Horsfall antigens, but in vivo injected trypan blue accumulated selectively to proximal tubular cells. Class I expression was high (84 to 95% of about 10^5 cells grown in Lab-Tek culture chambers were reactive with anti-class I antibody) in unstimulated endothelial, glomerular epithelial and tubular cells and was even higher (100%) after incubation with gamma-interferon for three days. Mesangial cells expressed class I considerably less in normal state (34%), but gamma-interferon induction upregulated it to 95%. Both the surface and intracytoplasmic expression of class I antigens were upregulated with three-day gamma-interferon treatment. Class II expression was low in all unstimulated cells (5 to 10%). The three-day gamma-interferon treatment upregulated both surface as well as cytoplasmic expression of class II antigens in all cell types.

Techniques for the isolation and culture of rat glomerular epithelial, glomerular mesangial, tubular and heart endothelial cells have been outlined by some investigators [1–4]. Characterization of the cultured cells has been based on morphology [5, 6], specific response to different cytotoxic agents [7], functional and metabolic experiments [8, 9] and, to a lesser degree, on the use of specific antibodies [10–12].

We have used these previously described isolation methods to attain purified populations of rat kidney glomerular epithelial, mesangial and tubular and heart endothelial cells. We have identified these different cell components by morphology and with monoclonal antibodies. The intracellular and surface expression of the major histocompatibility complex antigens has then been investigated by immunoperoxidase staining, fluorescence-activated cell sorter (FACS) analysis and by the *Staphylococcus aureus* rosette method for cell surface antigens.

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The information gained by this study, can be used in functional assays, for example, in the characterization of the importance of different target cell types in kidney allograft rejection. These isolated cell types can be used as target cells in natural- and lymphokine-activated killer cell assay as well as cytotoxic T-lymphocyte targets.

Methods

Isolation and culture of kidney components

The nucleus of DA ($RT1^a$) rats was obtained from Professor J. Gowans, Dunn School of Pathology (Oxford, UK). The rats were bred in our own colony and were regularly tested for the absence of positive intrastrain mixed lymphocyte culture.

Kidneys were removed from 5 to 10 week-old DA-rats and immediately put in cold PBS. The kidney cortex was separated carefully from the medulla with a scalpel, washed twice with PBS and pushed through a steel mesh with pore size of 250 μm with a syringe. The minced tissue was diluted with serum free MEM (Minimum essential medium, Gibco Limited, Paisley, Scotland, UK; D-valine added instead of L-valine to inhibit fibroblast growth; supplemented with 50 μg/ml gentamycin (Gibco), 2 μmol/ml glutamine (Gibco) and 5 μmol/ml HEPES-buffer). Tubular components were isolated by washing the tissue through a steel mesh with pore size of 100 μm; glomerular components were collected by filtrating the supernatant through a 75 μm steel mesh. The isolated components were washed twice with sera-free MEM.

After centrifugation, one part of the glomerular components was treated with type IV collagenase (Sigma Chemical Co., St. Louis, USA; 750 IU/ml in 5 ml of sera-free MEM, in +37°C and 5% CO_2 for 30 minutes, shook every 5 minutes). After collagenase treatment the components were washed with MEM containing 20% FCS and centrifuged with 200 g for five minutes.

Different cells were cultured in T-25 flasks (Nunclon, Copenhagen, Denmark) or in Lab-Tek chamber slides (Miles Scientific, Naperville, Illinois, USA). For tubular and glomerular epithelial cells (tubular and untreated glomerular components, respectively) culture flasks and slides were coated with collagen and fibronectin (Sigma; both 10 μg/ml in PBS) for at least one hour before culturing. Culture media with 10% FCS was used. For mesangial cells (enzyme treated glomerular components) uncoated culture dishes and 20% FCS media were used instead.

For the first two days (to secure the attachment of the cells onto plastic) all the components were cultured at 37°C and 5% CO_2 in a small volume (1 ml media in a T-25 flask) of cell growth media including 50 μg/ml gentamycin, 2 μmol/ml L-glutamine, 5 μmol/ml HEPES-buffer, 0.68 IU/ml insulin (40 IU/ml Actrapid, Novo Industri A/S, Copenhagen, Denmark), 30 μg/ml transferrin (Boehringer, Mannheim, FRG) and 10% or 20% of inactivated (56°C 30 min) FCS (Sera-Lab Limited, Sussex, UK) in MEM. On the third day, 5 ml of culture media was added and it was changed every second day from then on.

The mesangial and epithelial cell cultures were usually confluent after seven days and tubular cell cultures after nine days. Cell growth was checked daily with an upsidedown microscope (Olympus, Olympus Optical Co., Tokyo, Japan).

For detachment of the cells from bottles, 5 ml of versene (Gibco, 1:5000) was added. After incubation for 15 minutes in 37°C, the cells were washed through a 50 μm steel mesh to obtain a single cell suspension and washed once in MEM.

Glomeruli and tubular fragments were separated by using a combination of steel meshes with different pore sizes (100 and 75 μm, respectively). With this technique the glomeruli are practically free of capsules and vascular poles, and there is very little tubular contamination in the glomerular fraction. Similarly, the tubular compartment contains very few glomeruli as judged by phase contrast microscopy. To further confirm this separation, the rats were injected intraperitoneally with 200 mg/kg of trypan blue 48 hours prior to sacrifice and isolation of the cells [9]. Trypan blue accumulates selectively only to the proximal tubuli as confirmed in histologic sections. In cell suspensions the glomerular fraction was totally free of stained tissue, whereas 75% of the tubular fragments were strongly trypan blue positive, indicating that at least 3/4 of the tubular fraction contained proximal tubuli.

Isolation of endothelial cells

Four- to 12-day-old DA rats were used. A modification of the method of Kasten [1] was used. Rat hearts were minced with a scalpel and incubated three times in serum free MEM with 0.2 mg/ml of DNA:se (I, 600 IU/mg, Sigma) and 0.2 mg/ml of collagenase (183 U/mg, Worthington). After the first incubation (10 min at 37°C, magnetic stirring) the supernatant was discarded. Two more 15 minute incubations were performed and the supernatants were collected and filtered through a 50 μm steel mesh. The red cells were lysed with lysing reagent (ammonium cloride (NH_4Cl) 8.26 mg/ml, potassium carbonate ($KHCO_3$) 1.0 mg/ml and tetrasodium-EDTA (titriplex III) 0.037 mg/ml dissolved in distilled water) for five minutes in 37°C followed by two washings. The single cells were diluted in MEM with 10% of FCS and plated in T-25 tissue culture flasks or in Lab-Tek tissue culture chambers. After a 90 minute incubation the non-attached myocardial cells were discarded and the highly adherent endothelial cells were left in the flask. Endothelial cells usually grew to confluence in five days.

Immunoperoxidase stainings

Living cells cultured in chamber slides or cytosentrifuged (Cytospin, Shandon Elliot, London, UK) cell smears were fixed with acetone for five minutes. All subsequent procedures were carried inside a humidified box. The specimens were exposed for 30 minutes either to mouse monoclonal antibodies or to rabbit polyclonal antibodies (Table 1).

Table 1. Primary antibodies used in this study

Mouse monoclonals	
Anti-class I	MRC OX-18, (Serotec Ltd., Blackthorn Bices, UK)
Anti-class II	MAS 029, (Sera-Lab Ltd., Crawley Down, UK)
Anti-cytokeratin PKK1	Anti-human antibodies
Anti-vimetin	(gifts from Dr. Ismo Virtanen,
Anti-desmin	Dept. of Pathology, University of Helsinki, Helsinki, Finland)
Rabbit polyclonals	
Anti-brush-border	Anti-rat antibody (gift from Dr. Aaro Miettinen, Dept. of Bacteriology and Immunology, University of Helsinki, Helsinki, Finland)
Anti-Tamm-Horsfall	Anti-human antibody (gift from Dr. A. Miettinen)
Anti-factor VIII	Dakopatts, Copenhagen, Denmark

After incubation with the first antibody the specimens were washed with PBS and exposed to HISTOSTAIN:SP kit (Zymed Laboratories Inc., San Francisco, USA) according to manufactor's instructions. This kit utilizes a biotinylated second antibody, a horseradish peroxidase-streptavidin conjugate and a substrate (hydrogen peroxide)-chromogen (aminoethyl carbatsole) mixture to demonstrate antigen in cells or tissues.

Staphylococcus aureus *rosette assay*

The *staphylococcus aureus* rosette assay [13] was used to analyze the surface antigens of the cells isolated from rat kidney and heart. The cells were incubated with a 1:20 dilution of the indicated monoclonal antibody on ice for 30 minutes, washed twice, and incubated with staphylococcus (*Staphylococcus aureus* strain Cowan I) was received from Professor J. Wigzell, Department of Immunology, University of Uppsala (Uppsala, Sweden) for 30 minutes, washed twice, cytocentrifuged onto microscopic slides and finally stained with MGG. From the stained smears it was possible to differentiate between a rosette forming and non-rosette forming cell and to simultaneously observe the cellular morphology.

Fluorescence-activated cell sorter analysis

The cells were also tested by fluorescence-activated cell sorter (FACS) for their expression of class I and class II antigens. The cells were first exposed to a monoclonal antibody (Table 1) diluted 1:20, washed twice then exposed to a monoclonal fluorescein isotiocyanate conjugated anti-mouse antibody (GAM-FITC, Coulter Immunology, Hialeah, Florida, USA), washed twice, resuspended in PBS and run through the FACS.

Gamma-interferon stimulation

In some experiments the cells growing in flasks and on slides were stimulated with 100 U/ml rat gamma-interferon for three days (from Dr. P.H. v.d. Meide, Rijswijk, Netherlands).

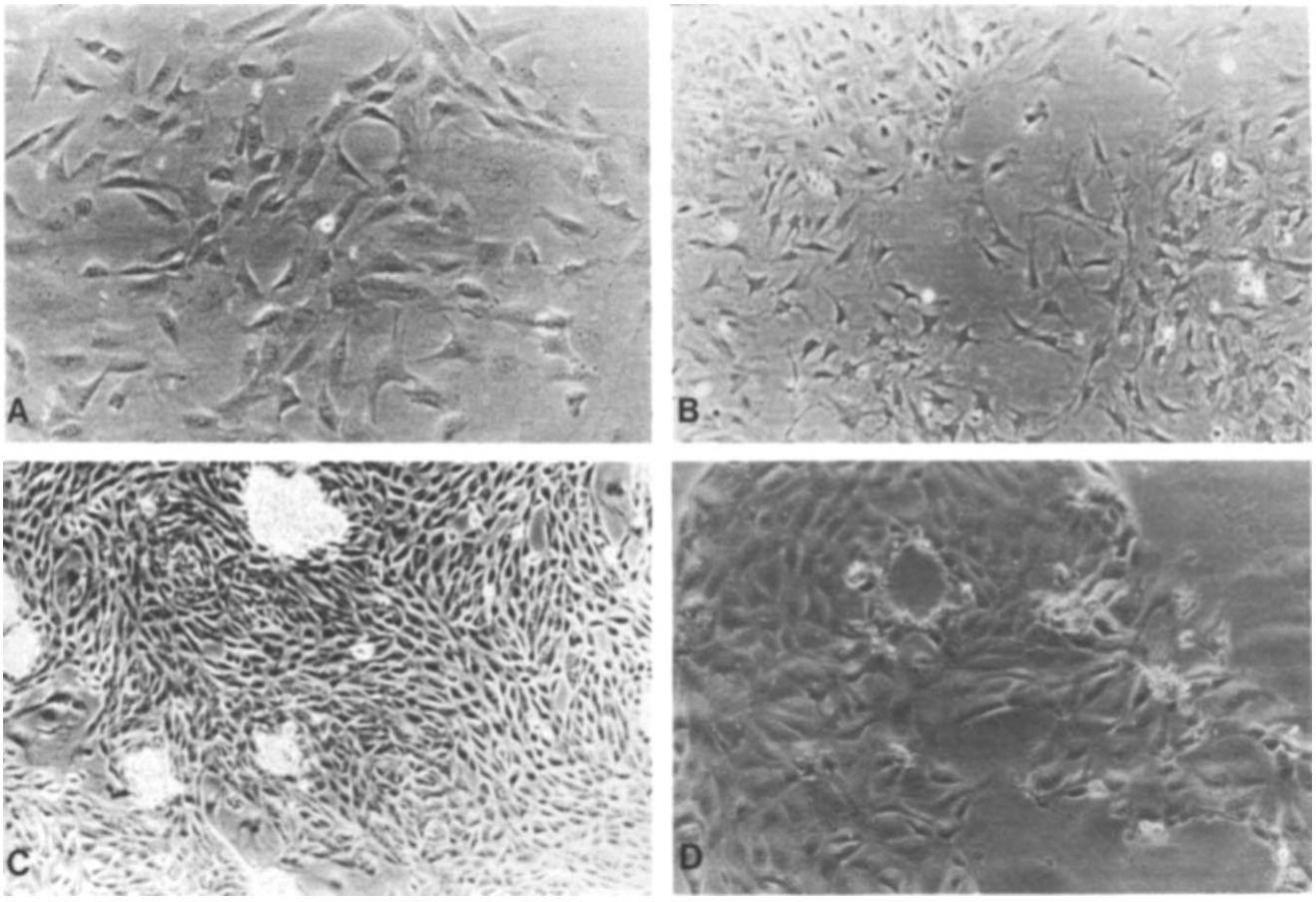

Fig. 1. *Different cell types cultured in T-25 flasks (phase contrast microscopy).* **A.** Endothelial cells before confluency (×13.6 magnification). **B.** Fusiform shaped glomerular mesangial cells (×6.8), six-day-old culture. **C.** Epithelial cells growing out of glomeruli (×6.8). **D.** Tubular cells in six-day-old culture (×6.8).

Table 2. Immunoperoxidase stainings of cytosentrifuged cell smears (percentages representing positive cells)

Antibody	Cell types			
	EC	GMC	GEP	TC
Anti-desmin 1:2	4	85	1	0
Anti-cytoceratin 1:20	0	5	12	30
Anti-LCA 1:100	0	8	0	1
Anti-brush border 1:200	0	12	40	55
Anti-Tamm-Horsfall 1:50	2	11	51	45

Abbreviations are: EC, endothelial cells; GMC, glomerular mesangial cells; GEP, glomerular epithelial cells; TC, tubular cells.

Results

Morphological criteria

Endothelial cells formed a typical monolayer in confluency (Fig. 1A). Mesangial cells were fusiform in shape (Fig. 1B), which fuse and overlap each other forming "hills and valleys" in extended culture. Typical epithelial morphology was seen with phase contrast microscopy in glomerular epithelial cell and tubular cultures (Figs. 1C and 1D, respectively): Cells were polygonal, and formed a tightly packed cobblestone-like monolayer in confluency. Judged by phase contrast microscopy mesangial cells had 5 to 15% contamination of epithelial morphology and a minor contamination of leucocytes.

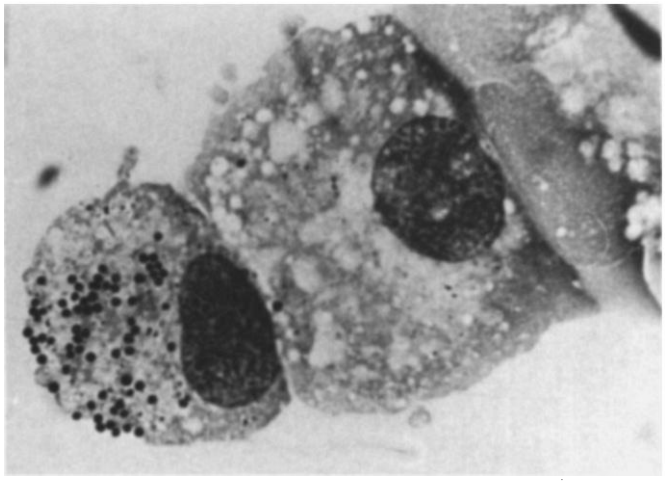

Fig. 2. *Staphylococcus aureus rosette with endothelial cells (EC).* One gamma-interferon stimulated EC reactive with anti-class II antibody and another non-reactive.

Antibody stainings

Endothelial cells stained only with anti-vimentin (97%) and anti-factor VIII (97%). Anti-desmin stained mesangial cells (80%). Furthermore, 5 to 10% of the cells in mesangial cell

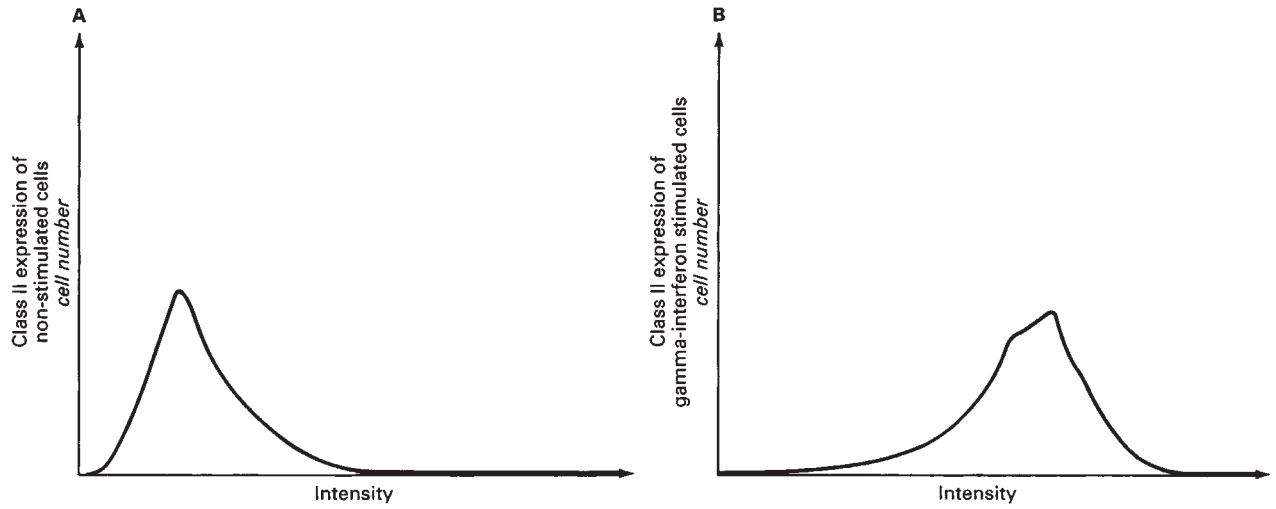

Fig. 3. *Fluorescent activated cell sorter analysis of glomerular epithelial cells.* **A.** Class II expression of non-stimulated cells. **B.** Class II expression of gamma-interferon stimulated cells. Shift to the right along the intensity axis describes the increase of positive (class II antigen expressing) cells compared to non-stimulated cells.

cultures stained positively with common leukocyte antibody (LCA), indicating that there was some white cell contamination (presumably glomerular macrophages) or a cross reactive antigen in our mesangial cell population. No positive reaction with LCA was found in other cell populations. We were unable to make a clear distinction between glomerular epithelial cells and tubular cells with anti-cytokeratin and anti-vimentin; however, the method of preparation enabled adequate separation of these two population of cells. Similarly, anti-brush border and anti-Tamm-Horsfall showed positive reactions in both populations (Table 2). Only trypan blue ingestion adequately identified the proximal tubular cells.

MHC-antigen expression

Unstimulated and gamma-interferon stimulated cells were tested for MHC-antigenicity with immunoperoxidase (IP) staining (both surface and intracytoplasmic reactivity), *Staphylococcus aureus* rosette assay and FACS-analysis (surface reactivity; Figs. 2 and 3, respectively).

Class I expression was high (84 to 95%) in unstimulated endothelial, glomerular epithelial and tubular cells in IP and FACS analyses and was even higher (100%) after a three-day gamma-interferon incubation (Table 3). Mesangial cells expressed class I antigen considerably less in normal state (34% in FACS; Table 4), but the three-day gamma-interferon induction increased it to 95%. Class I antigen was exposed on the cell surface of all the cell types (10 to 30%), the tubular cell expression being the highest. Surface expression was also upregulated in all cell types with the three-day gamma-interferon treatment (40 to 70%, Table 3).

Class II expression was low in all unstimulated cells (5 to 10%), being most intense with endothelial and mesangial cells. Epithelial and mesangial cells expressed class II antigen also on the cell surface (5 to 10%) but endothelial and tubular cells were surface negative. Gamma-interferon treatment vigorously upregulated cytoplasmic class II antigen expression of all the cell types (30 to 94%); concomitantly the surface expression of class II antigen increased to 30 to 60%.

Table 3. Class I and class II antigen expression of the different cell types (percentages representing positive cells)

Cell type	Class I surface	(1:1000) intracytop.	Class II surface	(1:1000) intracytop.
Endothelial				
Normal	10	95	2	10
Stimulated	40	100	30	94
Mesangial				
Normal	10	50	10	10
Stimulated	54	100	30	30
Epithelial				
Normal	20	95	5	5
Stimulated	50	100	40	70
Tubular				
Normal	30	90	0	5
Stimulated	70	100	60	55

IP-staining (Zymed, intracytoplasmic); Staphylococcus rosettes (surface).

Discussion

This study consists of two parts: (a) isolation of rat microvascular endothelial cells and the major cellular components of the nephron, and characterization of the purity of these cell populations, and, (b) investigation of whether and to what extent do these cell populations express class I and II MHC-antigens intracytoplasmically and on the cell surface in resting and gamma-interferon stimulated state. Endothelial cells were characterized by anti-factor VIII positivity [14]. Anti-desmin stained only cells with mesangial morphology [10]. In our mesangial cell cultures, 5 to 10% of cells were stained with anti-LCA. This is in agreement with Schreiner and Unanue [15], who suggested that these cells are mesangial phagocytes and estimated these LCA positive cells to form in 3 to 7% of the total mesangial cell population. Both glomerular epithelial and tubular cell populations expressed the brush border and Tamm-Horsfall antigens in immunoperoxidase stainings. Tamm-Horsfall protein has been generally found in the distal tubule. The anti-Tamm-Horsfall antibody we used was, however, an anti-

Table 4. Class I and class II antigen expression of the cell types (percentages representing positive cells), fluorescence activated cell sorter (surface)

Cell type	Class I	Class II
Endothelial		
Normal	94.2	27.2
Stimulated	96.4	94.7
Mesangial		
Normal	34.2	13.0
Stimulated	94.6	49.5
Epithelial		
Normal	86.2	5.6
Stimulated	97.7	94.8
Tubular		
Normal	84.7	1.9
Stimulated	73.3	49.6

human antibody and possibly crossreactive with rat glomerular epithelial and mesangial cells.

Ferry et al [14] demonstrated that cultured normal endothelial cells express 99% of class I and 12% of class II antigen. Frozen section studies have shown that class I and class II are present in the endothelial cells of rat heart cavity, in the capillaries and in small arteries and veins, but the antigens are absent from large arteries and veins [16]. Class I and class II antigens are also absent from rat kidney vascular endothelium of large vessels, but class II antigens are present in glomerular endothelial cells [17]. We found considerable class I antigen positivity in mesangial and epithelial cell populations although contraversial data exists [17].

Some investigators [18–20] have described that large amounts of class II antigens are seen inside proximal tubular cells but practically none of the antigen is exposed on the cell surface. In our tubular fraction, containing 75% proximal tubular cells, only 5% expressed class II intracellularly in immunoperoxidase staining in resting state, the surface expression being 0%. It has been reported that, using frozen sections Ia antigens can be localized to the lateral and basal cell membranes between the rat tubule epithelial cells [21]. These differences in Ia expression on cell surface can be explained by different methods used. Ia antigen expression in vivo represented by frozen sections may be different from the expression in vitro cultures. Bishop et al have been demonstrated that human renal tubular cells do not normally express HLA-DR antigens, but it is possible to induce increased expression of both class I and class II HLA antigens on them [3, 13]. It has also been reported that only rare canine renal tubular cells expressed class II antigen in native kidney [4].

Taken together we have demonstrated for the first time, using purified populations of rat microvascular endothelial cells and different cellular components of the nephron, that class I and II MHC antigens are present, although occasionally in only very minor amounts, on the surface of all these different cell types and that their presence on the cell surface can be upregulated by gamma-interferon. On the other hand, the antigen expression both in resting state and after gamma-interferon stimulation varies considerably from population to population. This observation offers a practical background to investigate the molecular mechanism of MHC expression in renal parenchyma, particularly whether the second signal in MHC antigen expression in response to gamma-interferon operates via the same or via a different messenger pathways in different cell types.

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